Thermal Stability of Hepatitis E Virus as Estimated by a Cell Culture Method

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ABSTRACT
Hepatitis E virus (HEV) is an increasingly recognized zoonotic pathogen. Transmission is suspected to occur from infected pigs or wild boars to humans through direct contact, environmental pathways, or contaminated food. However, the physical and chemical stability of HEV is largely unknown, because suitable cell culture methods for infectivity measurement are missing. Here, we developed a titration method using infection of the cell line A549/D3 with HEV genotype 3 strain 47832c and subsequent counting of focus-forming units by immunofluorescence, which allowed HEV infectivity measurements within a 4-log-dilution range. Long-term storage of HEV in cell culture medium at different temperatures indicated a phase of rapid virus inactivation, followed by a slower progression of virus inactivation. Infective HEV was detected up to 21 days at 37°C, up to 28 days at room temperature, and until the end of the experiment (56 days) with a 2.7-log decrease of infectious virus at 4°C. Heat treatment for 1 min resulted in moderate decreases of infectivity up to 60°C, 2- to 3.5-log decreases between 65°C and 75°C, and no remaining virus was detected at temperatures of ≥80°C. Heating for 70°C resulted in a 3.6-log decrease after 1.5 min and the absence of detectable virus (>3.9-log decrease) after 2 min. The data were used to calculate predictive heat inactivation models for HEV. The results may help estimate HEV stability in the environment or food. The established method may be used to study other aspects of HEV stability in the future.

IMPORTANCE
In this study, a cell culture method was developed which allows the measurement of hepatitis E virus (HEV) infectivity. Using this system, the stability of HEV at different time-temperature combinations was assessed, and a predictive model was established. The obtained data may help estimate HEV stability in the environment or food, thus enabling an assessment of the relative risks of HEV infection through distinct routes and by distinct types of food in the future.

Hepatitis E virus (HEV) is the causative agent of acute hepatitis worldwide (1). Large outbreaks of hepatitis E have been repeatedly described from developing countries in Asia and Africa, e.g., in Nellore, India, with an estimated 23,915 affected persons and an estimated 314 deaths in 2008 (2). In industrial countries, mainly sporadic cases of acute hepatitis E are recognized (3). The numbers of notified hepatitis E cases have significantly increased in several European countries during the last few years (4, 5). In addition, chronic HEV infections in immunocompromised transplant patients constitute an emerging problem (6).

There are many different possible transmission pathways of HEV. HEV genotypes 1 and 2 exclusively infect humans and are mainly transmitted by contaminated drinking water (1). In contrast, HEV genotypes 3 and 4 are zoonotic viruses infecting humans and several animal species, like pigs, wild boars, deer, and rabbits (7). Direct transmission by contact with infected animals and foodborne transmission through meat and meat products produced from infected animals are considered the main infection routes for humans (7). However, parenteral transmission by blood transfusion or administration of blood products and environmental contamination have also been considered (8).

HEV is a nonenveloped icosahedral virus with a single-stranded RNA genome of positive polarity (7). However, recent experiments with HEV from tissue culture and serum samples from patients indicated the presence of an additional fraction of HEV containing lipid-associated membranes (9). The stability of HEV against physical and chemical treatments has been only poorly characterized so far. One major reason for this is the lack of suitable cell culture assays for measuring HEV infectivity (10). However, the recent development of novel cell culture techniques (11) and the isolation of more efficiently replicating HEV strains (12, 13) may overcome these problems. Other approaches, such as seroconversion after experimental inoculation of pigs for an assessment of infectivity (14) or capsid integrity assays as proxies for infectivity (15) after heat treatment of HEV, have been considered.

This study aimed at developing a cell culture-based titration system for an assessment of HEV stability using the recently cell culture-adapted genotype 3 strain 47832c (13). The system should thereafter be used for the assessment of HEV stability at different temperatures and time periods. The obtained data will be used to estimate the stability of HEV in the environment or food, thus contributing to the assessment of the relative risk of HEV infection through distinct routes. In addition, the established method may be used to study other aspects of HEV stability in the future.

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MATERIALS AND METHODS

Cells and viruses. HEV strain 47832c is a cell culture-adapted genotype 3 strain originally isolated by inoculation of a serum sample from a chronically HEV-infected patient onto A549 cells (13). By subsequent passage of these infected cells, a persistently HEV-infected A549 cell line was produced, which continuously releases HEV particles into the cell culture medium (13). Another cell line, designated A549/D3, was generated from noninfected A549 cells by seeding of a single cell and growing out to a cellular clone. All cell lines and viruses described here are available by request from the authors for academic research.

Preparation of HEV samples. The persistently HEV-infected A549 cells were grown in T25 flasks in minimum essential medium (MEM) Eagle complemented with 1% nonessential amino acids, 1% glutamine, 0.5% gentamicin, and 5% fetal calf serum (FCS; all cell culture media by PAN-Biotech GmbH, Germany) at 37°C and 5% CO₂ in a humidified incubator. After 7 days of incubation, the supernatant was removed, centrifuged to remove cells, and stored at −80°C. The cells were split in half and grown for another 7 days, when the supernatant was collected as described above. The stored supernatants were tested by HEV-specific reverse transcription-quantitative PCR (RT-qPCR), according to Schielke et al. (15), and those containing large amounts of HEV RNA were pooled. The resulting virus stock solutions contained 7.7 × 10⁶ HEV genome copies/ml and 1.1 × 10⁹ focus-forming units (FFU)/ml (determined as described below) in the first series of experiments, and 1.3 × 10⁹ HEV genome copies/ml and 7.4 × 10⁶ FFU/ml in the second series of experiments.

Long-term storage and heat treatment. For long-term storage experiments, 100-µl aliquots of the virus stock solution were pipetted into 1.5-ml Eppendorf tubes and stored at different temperatures (37°C in a temperature-controlled incubator, room temperature [approximately 22°C] in a box protected from light at the laboratory bench, and 4°C in a temperature-controlled refrigerator). After 0, 1, 2, 3, 4, 7, 10, 14, 21, 28, and 56 days, two of the tubes were removed in each experiment and stored at −80°C. For short-term heat treatments, 100-µl aliquots of the virus stock solution were pipetted into 1.5-ml Eppendorf tubes and held at room temperature. The cavities of a Thermomixer Comfort 1.5-ml tube block (Eppendorf, Germany) were filled with water for rapid heat transfer and preheated at the designated temperature. The temperature was additionally checked using a conventional thermometer. Two aliquots each were placed into the preheated thermomixer for the indicated time period, subsequently placed on ice water for rapid cooling, and thereafter were frozen at −80°C.

Titration of treated HEV samples. A549/D3 cells were seeded into 96-well plates (1 × 10⁴ cells in 100 µl per well) in MEM Eagle complemented with 1% nonessential amino acids, 1% glutamine, 0.5% gentamicin, and 10% FCS at 37°C and 5% CO₂ in a humidified incubator. The medium was removed and replaced with new medium at day 7 after seeding. After incubation for an additional 3 days, the medium was removed, and the confluent cell monolayers were washed twice with 200 µl of phosphate-buffered saline (PBS). A dilution series of the samples was performed in a separate 96-well plate. Briefly, 90 µl/well cell culture medium (as described above, but without FCS) was added to rows B to G of this plate. The samples were thawed at 37°C and added to row A (100 µl in each well). A 10-fold virus dilution series was prepared by transferring 10 µl/well from row A to row B, mixing 3 times, and repeating this procedure through row F. Row G served as a negative control, receiving no virus, and row H was inoculated with 100 µl/well of the virus stock solution as a positive control. The dilutions were transferred to the cells, incubated for 1 h at room temperature, and thereafter were replaced by 200 µl/well cell culture medium containing 5% FCS. After incubation for 7 days at 34.5°C and 5% CO₂ in a humidified incubator, the medium was removed, exchanged with fresh medium, and incubated as described above for another 7 days. Thereafter, the medium was removed, replaced by 100 µl/well of an acetone-methanol solution (1:1; −20°C) for fixation, and incubated for 30 min at 4°C. The solution was thereafter removed, and the plate was dried at room temperature and stored at 4°C until analysis by immunofluorescence.

Immunofluorescence test. The fixed and dried cell culture plates were washed one time with 200 µl/well PBS. Thereafter, 200 µl/well PBS supplemented with 1% FCS was added and incubated for 30 min at 37°C. After removing the solution, 50 µl/well of a 1:500 dilution of an open reading frame 2 (ORF2)-specific HEV-specific rabbit hyperimmune serum (kindly provided by R. Ulrich, Friedrich Loeffler Institute, Germany) in PBS supplemented with 1% FCS was added. After incubation for 1 h at 37°C, the cells were washed twice with 200 µl/well PBS and 50 µl/well of a 1:1,000 dilution of fluorescein isothiocyanate (FITC)-conjugated anti-rabbit IgG (Sigma, Deisenhofen, Germany) in PBS supplemented with 1% FCS was added. After incubation for 1 h at 37°C, cells were washed twice with 200 µl/well PBS and once with 200 µl/well distilled water. The plates were dried and stored in the dark at room temperature. Cells were analyzed using the Zeiss Axio Observer Z1 microscope (Zeiss, Jena, Germany).

Quantification of virus infectivity. All wells were manually observed for specific fluorescence, and the presence of fluorescent foci was recorded. A fluorescent focus was defined as a minimum of two adjacent cells showing clear intracytoplasmic fluorescence. The number of fluorescent foci was counted in the highest dilution showing fluorescence, and the respective focus-forming units (FFU) were calculated. The limit of detection (LoD) was 1 FFU. The mean value of log₁₀-transformed FFU values was calculated per time point and temperature using the Excel software.

Predictive modeling. In a first step, we compared the applicability of 4 different nonlinear inactivation equations (Geeaerdt without Nres, Mafart, Albert, and Weibull) to fit the observed data. Based on this preanalysis, a primary regression model was generated for each of the four temperature conditions (4, 22, 37, and 70°C) by fitting the Mafart equation (16) through all available data points above the LoD using the software PMM-Lab (17). The Mafart equation was selected, as the data showed nonlinear inactivation characteristics at all temperature regimes. The relationship between the estimated model parameter delta (time to first decimal reduction) and temperature was then visually inspected. This analysis led to the finding that for the temperature range of 4°C to 37°C, this relationship could be modeled by the equation \( \text{SQRT} \left( \frac{a}{b \times \text{temperature}} \right) \), where \( \text{SQRT} \) is the square root and \( a \) and \( b \) are free parameters estimated through the fitting procedure. A global predictive model was then generated by fitting the combined empirical Mafart equation through all available experimental data from 4°C to 37°C, including those below the LoD, in a one-step fitting procedure applying the maximum-likelihood-based approach proposed by Lorimer and Kiermeier (18). The generated specific and global predictive models were finally exported as PMFX files (see http://dx.doi.org/10.13140/RG.2.1.1555.3044 and http://dx.doi.org/10.13140/RG.2.1.4701.0320). These PMFX files are in accordance with the PMF-ML guidance for a software-independent representation of mathematical models (http://sourceforge.net/projects/microbialmodelingexchange/) and further include the model’s parameter estimates and metadata, the raw experimental data, and the description of the model’s applicability range.

RESULTS

Establishment of a cell culture method for titration of HEV infectivity. A cell culture system was established for titration of HEV infectivity. It is based on infection of the cell line A549/D3 with the cell culture-adapted genotype 3 strain 47832c. The cell line A549/D3 was generated by growing out single cells from the cell line A549 to clonal cell lines. One of these clonal cell lines, designated A549/D3, was shown to be more susceptible to HEV strain 47832c infection than the parent A549 cell line (not shown). Replication of HEV did not cause any cytopathic changes in the cell line. Therefore, immunofluorescence staining of HEV-infected cells using an HEV-specific rabbit hyperimmune serum was used...
to count fluorescent foci, indicating replication of HEV. Infection experiments indicated that fluorescent foci could be detected by the use of dilutions of up to 1:10,000 of the virus stock (Fig. 1).

HEV infectivity after long-term storage at different temperatures. Aliquots of cell culture supernatant containing the HEV strain 47832c were stored at 4°C, room temperature (22°C), or 37°C and subsequently frozen at different time points within a 56-day-long period. The decrease in infectivity was assessed and described by determining the reduction in focus-forming units (FFU) using the developed titration method. In all cases, the infectivity decreased relatively rapidly within a first phase, followed by a slower decrease in a second phase (Fig. 2). At 4°C, a mean decrease of 1.6 log FFU occurred within the first 10 days, followed by another 1.1-log decrease until day 56, resulting in a mean total decrease of 2.7 log over the whole period (Fig. 2A). At room temperature, the average concentration decreased by 1.7 log FFU within the first 7 days, followed by a further 2.0-log decrease until day 28 (Fig. 2B). No remaining infectivity could be detected after storage at room temperature for 56 days, corresponding to a decrease in viral infectivity of at least 3.7 log FFU. At 37°C, a mean decrease of 2 log FFU occurred within the first 4 days, followed by a further 1.4-log decrease until day 21 (Fig. 2C). No remaining infectivity could be detected after storage at 37°C for 28 days, corresponding to a decrease of at least 3.3 log FFU. An independent experiment was performed by storage of another virus stock preparation at 4°C for 28 days, and subsequent titration on cells with a higher passage number. As evident from Fig. 2A to C (gray triangles), the results verify the observations of the previous experiment.

HEV infectivity after short-term heating. The effect of short-term heating was analyzed by heat treatment of cell culture supernatant containing HEV strain 47832c at different temperatures for 1 min. As shown in Fig. 3, only small decreases in infectivity (<1.3 log) occurred when the strain was heated to 60°C. Heating at 65°C, 70°C, and 75°C reduced infectivity on average by 2.6, 2.9, and 3.4 log FFU, respectively. No infectivity could be detected after heating at temperatures of ≥80°C for 1 min, corresponding to reductions of at least 3.5 log FFU. An independent experiment was performed by heating another virus stock preparation at 60°C, 65°C, 70°C, 75°C, and 80°C for 1 min and subsequent titration on cells with a higher passage number. As evident from Fig. 3 (gray triangles), the results are similar to those of the previous experiment; however, no remaining virus could be detected in this experiment after heating at 75°C for 1 min.

HEV infectivity after heating at 70°C for different time periods. In order to investigate the effect of different heat treatment regimens on HEV infectivity, the cell culture supernatant containing HEV strain 47832c was heated at 70°C for 10 s up to 10 min. As shown in Fig. 4, the infectivity decreased rapidly up to 2.9 log FFU within 50 s. After 90 s at 70°C, a decrease in infectivity of >3 log FFU was observed. No infectious virus could be detected after heating for 2 min and longer, corresponding to reductions of at least 3.9 log FFU. An independent experiment was performed by heating another virus stock preparation at 70°C for 0, 10, 30, and 60 s and subsequent titration on cells with a higher passage number. As evident from Fig. 4 (gray triangles), these results confirm the observations of the previous experiment.

HEV infectivity reduction model and its application. The measured experimental reduction in HEV infectivity from constant-temperature treatment regimens at 4°C, 22°C, 37°C, and 70°C could be used to generate temperature-specific primary models (Fig. 5A) with good accuracy (all root mean square errors [RMSE], <0.45 log FFU). In addition, a global model was generated that was capable of predicting the temperature-dependent reduction in HEV infectivity for any time-temperature combination in the range of 4°C to 37°C for up to 56 days. All estimated model parameters are provided in detail in a PMFX file (see http://dx.doi.org/10.13140/RG.2.1.1555.3044 and http://dx.doi.org/10.13140/RG.2.1.4701.0320). The RMSE of 0.35 log FFU (based on all experimental data above the LoD) again indicates a good accuracy of the generated model. This model can thus be used to predict the reduction in HEV infectivity in liquid and transparent solutions for any temperature treatment in a given range of applications. In addition, the time needed to achieve a desired reduction in infectivity can be predicted. For example, based on this

FIG 1 Infection of A549/D3 cells with HEV genotype 3 strain 47832c. The cells were infected with 10-fold dilutions of the virus and analyzed 14 days later by immunofluorescence using a rabbit anti-HEV hyperimmune serum. Noninfected A549/D3 cells are shown as a negative control; a light-phase-contrast picture of these cells is shown in the upper right corner. undil., undiluted.
model, it will take 19.6 days, on average, to achieve a 3-log FFU reduction at 30°C.

DISCUSSION

Data on the stability of HEV are urgently needed to enable an assessment of the infection risk through specific virus transmission routes and to elaborate suggestions to prevent HEV infection. The assessment of HEV stability was hampered in the past by the lack of convenient systems for measuring HEV infectivity. Inoculation experiments with heat-treated HEV-containing material into susceptible animals provided important data in the past (14, 19). However, animal experiments are expensive, difficult to perform, and ethically problematic, thus limiting those experiments to a very limited number of temperature-time combinations. Capsid integrity assays using RNase treatment followed by RNA extraction and RT-PCR can be applied to a higher number of samples (15). However, those assays may overestimate infectivity, as RNA may also be protected by a slightly damaged capsid showing no infectivity. Therefore, cell culture-based assays may provide the best alternative, enabling a direct measurement of infectivity combined with the possibility of higher throughput of samples (10). After a long time, in which appropriate cell culture systems for HEV were not available, the isolation of more efficiently replicating HEV strains (12, 13) now enables the development of suitable cell culture-based titration systems.

The system developed here for the assessment of HEV infectivity is based on HEV genotype 3 strain 47832c, which had been originally isolated from a chronically infected immunosuppressed transplant patient (13). This strain was chosen because it was previously shown to efficiently replicate in A549 cells and it could be repeatedly passaged in this cell culture. Moreover, a cell line persistently infected with this HEV strain was available, which enables

![Graphs showing HEV infectivity reduction at different temperatures](Figure 2)

**Figure 2** Reduction in HEV infectivity by long-term storage at different temperatures. Cell culture-derived aliquots containing HEV genotype 3 strain 47832c were stored at 4°C (A), room temperature (B), or 37°C (C), and the remaining infectious virus was analyzed in two replicates at different time points by titration on A549/D3 cells. The numbers of counted fluorescent foci of each replicate are shown in focus-forming units (log scale) by black circles, and the mean numbers are indicated by horizontal lines. The numbers of focus-forming units of replicates from selected time points determined in an independent second experiment are shown by gray triangles.
continuous production of infectious HEV for use in virus stability tests. In addition, the sub-cell line A549/D3 could be selected, which was shown to be susceptible to infection with several dilutions of this HEV strain. HEV genotype 3 is the most frequently detected HEV type in patients in Europe (20), and strain 47832c has been shown to be closely related to HEV strains from animals and diseased humans in Germany (13). Therefore, the strain may reflect the typical characteristics of HEV strains circulating in Europe. However, as it has been isolated from a chronically infected patient after extended virus persistence in the host, it cannot be excluded that specific mutations are present that influence its stability and replication efficiency. For instance, a specific insertion in open reading frame 1 of this strain has been considered to be responsible for efficient cell culture growth (13). Additional titration systems using other HEV strains should be developed in the future to assess the natural variability of HEV stability.

The applicability of the developed cell culture system is shown here in long-term storage and short-term heating experiments with HEV. Although the resulting data set is still small, it can be used for comparison with other studies and to generate a first predictive model for the inactivation of HEV in virus stock solutions. Systematic data on long-term stability of HEV previously were only generated using capsid integrity tests (15). In that investigation, wild boar liver homogenates containing an HEV strain closely related to strain 47832c were treated very similarly to those in our protocol, but the amount of RNase-protected HEV genomes was assessed in contrast to the amount of infectious virus. Interestingly, in both assays, a phase of rapid degradation/inactivation was followed by a phase of slower degradation/inactivation. However, it is evident from a comparison of the data that the RNase-protected genome degrades much more slowly than the reduction in infectivity measured in our assay. For instance, incubation at 4°C for 56 days resulted in a decrease of only approximately 0.5 log genome equivalents (15), whereas the infectivity decreased under the same conditions by approximately 2.7 log units, based on our measurements. Incubation at 37°C for 21 days resulted in an approximately 1.5-log decrease in the viral genome number, whereas the infectivity decreased by at least 3.3 log units. It has also been shown for related viruses, such as caliciviruses, that (RNase protection-based) RT-PCR analyses overestimate the stability of viruses compared to infectivity assays (21). Although the data yielded from our experiments may indicate that HEV is not as stable under long-term storage conditions as previously suspected, it still has to be considered that it remains infectious for several weeks. Therefore, HEV contamination of water, food, or the environment may persist for a long time, depending on the temperature and other distinct factors not yet analyzed in detail.

The sensitivity of HEV to short-term heating was investigated previously using capsid integrity assays (15), cell culture assays (22, 23, 24), and animal inoculation experiments (14, 19). Again, the capsid integrity assay indicated much higher heat resistance than that with the other systems. A comparison of our data to those of other cell culture studies is difficult, as the tested temperature-time combinations are not identical. However, most of these studies indicate that short-term heating to 56°C or 60°C cannot inactivate HEV (23, 24), which is in concordance with our data showing only minor decreases in infectivity after 1 min of heating at these temperatures. Longer incubations, however, may have stronger effects. For instance, Huang et al. (22) reported complete inactivation of an HEV genotype 1 strain after heating at 56°C for 30 min. Studies using seroconversion as a readout of infectious virus after experimental inoculation of pigs confirm the stability of HEV at 56°C. Feagins et al. (19) showed that heating for 1 h at 56°C did not completely inactivate HEV present in a pig liver homogenate, whereas heating for 5 min to 71°C was sufficient for inactivation. Barnaud et al. (14) showed that treatment of an HEV-containing liver paté-like preparation at 71°C for 10 min was not sufficient for HEV inactivation, whereas 20 min of incubation at this temperature completely inactivated HEV. Our data indicate that HEV infectivity rapidly decreases at temperatures of >65°C. However, residual virus was detectable after treatment for 1 min at 75°C and after treatment for 90 s at 70°C. The non-log-linear shape of inactivation curves under all temperature conditions indicates that the speed of HEV inactivation decreases over time. Therefore, a general recommendation to heat food at 70°C.
for 2 min might not be sufficient for complete HEV inactivation, especially if large amounts of virus are present.

From the experimental data generated in this study, we were able to create a first predictive model for inactivation of HEV applicable in the temperature range from 4°C to 37°C and covering up to 56 days. In addition, a primary HEV inactivation model was generated, allowing the prediction of the impact of heat treatment at 70°C for up to 90 s. The models will enable the prediction of HEV inactivation in the tested matrix at time-temperature combinations not tested experimentally. All models including the underlying experimental data were made available as PMFX files, which allow the software-independent representation of predictive models and therefore guarantee that generated models can easily be validated, deployed, and updated. Although the models showed good accuracy, as indicated by the low RMSE values, they are still based on a small data set. Further inclusion of additional data may therefore be desirable in the future.

Generally, a comparison of the data from heat stability testing of HEV is difficult, as it is dependent on the properties of the strains used, the effects of the matrix used, and the system applied to analyze the remaining infectivity. As the dose-response relationship of HEV is not known, it is also not clear which level of infectivity reduction would be necessary in order to securely prevent infection and disease in humans. However, the generation of more-detailed data on the infectivity reduction for different HEV strain-matrix combinations would clearly increase our understanding of HEV stability in the environment or food. By this, an assessment of the relative risks of HEV infection through distinct routes and by distinct types of food will become possible. The cell culture-based titration method developed here has the potential to facilitate such detailed analysis in the future.

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